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# Influence of dietary restriction on immunologic function and renal disease in (NZB × NZW)$F_1$ mice

(calories/autoimmunity/longevity/immunity/histopathology)

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**ABSTRACT** In (NZB × NZW)$F_1$ (B/W) mice, moderate caloric intake [10 kcal (41.8 kJ) per day] from the time of weaning was associated with maintenance of lower body weight, greater capacity of spleen cells to be stimulated with T-cell mitogens, and better preserved capacity to generate cytotoxic cells in response to *in vitro* and *in vivo* stimulation with allogeneic tumor cells. Plaque-forming cell response to sheep erythrocytes was also well maintained in animals on the restricted diets when sensitization was accomplished either *in vitro* or *in vivo*. Spontaneous suppressor cell activity against plaque-forming cells that developed in controls did not appear in the mice on the restricted diet. Significantly less circulating antibody to native DNA was present in the blood of mice 10 months of age when their dietary intake had been restricted. Histological analysis revealed that the development of renal disease and the deposition of gamma globulin in the glomerular capillaries was markedly inhibited in the mice on restricted diets. Dietary restriction from the time of weaning thus appears to prolong significantly the life of autoimmunity-prone (NZB × NZW)$F_1$ male and female mice and to alter lymphoid cell immune function, thereby decreasing the autoimmune processes and immunological assault associated with progressive renal disease in these animals.

Over the past several years, we have analyzed the influence of diet on immunologic functions in mice, rats, and guinea pigs (1). Investigations with the autoimmunity-susceptible NZB strain of mice revealed that diets relatively high in fat and low in protein and fiber accelerated development of autoimmune disease and shortened life-span. Conversely, diets high in protein and fiber and low in fat delayed development of autoimmunity and prolonged life-span (2, 3). Furthermore, chronic protein restriction with normal calorie intake from the time of weaning in NZB mice interfered with the loss of T-cell-mediated immune functions and decreased hypergammaglobulinemia, thymic involution, and splenomegaly (4). The low-protein diets also retarded moderately the development of autoimmune disease but did not prolong life of NZB mice (4). Relatively short-term, chronic, moderate protein restriction with normal calorie intake in rats (5), mice (6), and guinea pigs (7), inhibited antibody production but permitted expression of T-cell-mediated immunity. Extreme dietary protein deficiency inhibited both production of antibody and expression of T-cell-mediated immunities. Similar influences were obtained with chronic single amino acid restrictions (8). Walford *et al.* (9) showed that, in long-lived strains of mice, calorie restriction early in life prolonged immunologic vigor as well as survival.

We have recently reported that dietary restriction, initiated both from the time of weaning and at an adult age, prolongs significantly the life-span of short-lived, autoimmunity-prone (NZB × NZW)$F_1$ (B/W) mice (10, 11). Dubois *et al.* (12) reported that feeding of low-phenylalanine or low-tyrosine diets prolonged the life of B/W mice and prevented development of renal diseases. The latter investigators have also confirmed our findings of prolongation of life by dietary restriction and found this influence to be associated with decreased deposition of immune complexes in the kidneys; however, they found no influence on the immunological parameters (e.g., antinuclear antibody) or on the titer of the xenotropic virus, which has been associated with this disease (13).

This paper describes investigations that analyze further the influence of dietary restriction on B/W mice. We will show that restriction of diet from the time of weaning interferes dramatically with development of renal disease, as revealed histologically. This diet retards deposition of gamma globulin in the glomerular capillaries and decreases circulating antibody to native DNA. In addition, dietary restriction delays the decline of certain T-cell based cellular immunities *in vivo* and *in vitro*. Finally, dietary restriction interferes with capacity to generate a spontaneous suppressor cell influence on plaque-forming cell (PFC) immune response to sheep red-blood cells (SRBC) induced *in vitro*, which usually develops in well-fed B/W mice toward the end of life.

## MATERIALS AND METHODS

**Animals.** Inbred 3- to 4-week-old male and female B/W mice, produced at the University of Minnesota mouse colony by mating NZB and NZW strains, were used. The mice were housed under standard conditions that included controlled humidity and temperature and alternate light and dark cycles, as described (4, 10).

**Diets.** Composition of diet, source of ingredients, method of preparation, and feeding procedures as well as housing of animals were identical to that described previously (4, 10). Briefly, the diet was 22% casein, 33% dextrose, 33% starch, 5% corn oil, 4% mineral mixture, 2% vitamin mixture, and 1% agar. However, animals on the restricted (low-calorie) diet [10 cal (41.8 kJ) per day] were housed singly and were given supplements of salt and vitamins in rations equal to the amounts given to the animals fed the normal diets (20 cal per day). Exact amounts of food, either 5 g (approximately 20 cal) or 2.5 g (approximately 10 cal), were provided at the same time each day between 8 and 10 a.m., during the early part of the light cycle.

Abbreviations: B/W, (NZB × NZW)$F_1$; PFC, plaque-forming cells; SRBC, sheep red blood cells; calorie, the nutritionist's calorie = 1 kcal = 4.18 kJ; Con A, concanavalin A.

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Table 1. Cytotoxic cell-mediated immune response of B/W spleen cells immunized either *in vitro* or *in vivo* with EL-4 cells*

| Age, mos. | Diet, cal/day | Percentage $^{51}$Cr release at 4 hr | | | |
|---|---|---|---|---|---|
| | | Immunized *In vitro*† | | Immunized *In vivo*‡ | |
| | | 1:4† | 1:8 | 1:25 | 1:100 |
| 4 | 20 | 44 ± 3 | 58 ± 5 | 24 ± 2 | 43 ± 3 |
| | 10 | 29 ± 2 | 43 ± 4 | 18 ± 3 | 50 ± 5 |
| 10 | 20 | 10 ± 2 | 17 ± 4 | 6 ± 2 | 12 ± 3 |
| | 10 | 49 ± 3 | 64 ± 3 | 16 ± 3 | 40 ± 9 |

* Three to five individual spleens were tested in each group, with triplicate samples. Results shown as mean ± SEM.
† Immunized *in vitro* with mitomycin-treated EL-4 tumor cells. Ratios are target cells to lymphocytes.
‡ Immunized *in vivo* with EL-4 tumor cells ($10^7$, intraperitoneally) 10 days prior to testing. Ratios are target cells to lymphocytes.

**Tumor Cells.** EL-4 lymphoma cells ($H\text{-}2^b$) were maintained by weekly serial intraperitoneal injection of EL-4 cells into syngeneic C57/BL/6 hosts.

**Response to *In Vivo* and *In Vitro* Immunization with SRBC.** Details of the *in vivo* assay were described (14). Each mouse was injected intraperitoneally with 0.2 ml of a 20% suspension of SRBC (Wilfer Farms, Minneapolis, MN); 96 hr later, the spleens were removed and the viable cell concentration was adjusted to $10^7$ cells per ml. A modified Jerne PFC assay (15) was used to measure PFC response. The immunizing dose of SRBC was found, in preliminary studies, to be optimal for B/W mice.

Spleen cells free of erythrocytes were prepared to measure *in vitro* responses and were cultured in plastic petri dishes (Falcon, no. 3001) at a concentration of $10^7$ cells per ml with SRBC (Grand Island Biological Co., Grand Island, NY) in RPMI-1640 medium with 10% fetal calf serum by the method of McCarthy and Dutton (16). The cultures were fed daily and harvested on day 5 (peak response) for measurement of PFC.

To determine suppressor cell activity in this system, spleen cells were set up 36 hr earlier either with medium alone or with concanavalin A (Con A) (2.5 $\mu$g/ml). After being washed twice with RPMI-1640 medium, these cells were resuspended in fresh culture medium at the required cell density and mixed with fresh spleen cells ($10^7$) and SRBC (17). Each culture was set up in duplicate, and cells from each culture dish were plated in agar in duplicate.

**Cytotoxic Response to Immunization with Allogeneic Antigens.** For *in vivo* immunization, the mice were injected peritoneally with an optimal dose ($5 \times 10^6$) of EL-4 lymphoma cells. Ten days after immunization, the mice were sacrificed and the cytotoxic activity of their spleen cells was determined in a microplate $^{51}$Cr-release assay system (18).

*In vitro* allogeneic immunization was performed in plastic multiwell dishes (FB16-24TC, Linbro Scientific, New Haven, CT) by a modification of the method of Hodes *et al.* (19). Spleen cells ($4 \times 10^6$) from B/W mice were mixed with $0.5 \times 10^6$ mitomycin C-treated (25 $\mu$g/ml, 37°, 30 min) spleen cells from C57/BL/6 mice in 2 ml of RPMI-1640 medium with 10% fetal calf serum and 50 $\mu$M 2-mercaptoethanol, nonessential amino acids, and pyruvate, and incubated at 37° in an atmosphere of 5% $CO_2$/95% air for 5 days to obtain maximal response. Triplicate wells were then pooled and the cytotoxic activity of the viable cell population was determined (19).

**Measurement of DNA Antibody.** DNA-binding capacity of the serum of individual mice was measured by a micromodification of the technique described by Wold *et al.* (20, 21). The results were expressed as the mean percentage of $^{125}$I-labeled DNA bound by antibody and thus precipitable by ammonium sulfate. The values obtained were corrected for nonspecific precipitation in normal mouse serum. Renal biopsy specimens were analyzed by light and fluorescent microscopy (22, 23). Frozen sections were stained with goat antibodies to mouse IgG, IgM, and C3.

Table 2. *In vivo* and *in vitro* PFC response to SRBC in different age groups of B/W mice fed either 20 or 10 cal per day

| Age, mos. | Cal/ day | PFC* (*in vivo* immunization) | | PFC† (*in vitro* immunization) | |
|---|---|---|---|---|---|
| | | Per $10^6$ cells | Per spleen | Per $10^6$ cells | Per culture |
| 3 | 20 | 3108 ± 351 | 394,421 ± 41,639 | — | — |
| 3 | 10 | 2975 ± 147 | 186,942 ± 23,015 | — | — |
| 4 | 20 | — | — | 285 ± 153 | 860 ± 310 |
| 4 | 10 | — | — | 303 ± 128 | 1245 ± 265 |
| 6 | 20 | — | — | 141 ± 22 | 450 ± 79 |
| 6 | 10 | — | — | 249 ± 76 | 883 ± 213 |
| 10 | 20 | 70 ± 12 | 6,790 ± 1,125 | 34 ± 14 | 123 ± 13 |
| 10 | 10 | 960 ± 362 | 100,931 ± 25,968 | 294 ± 8 | 1403 ± 99 |

* Mean ± SEM from four mice immunized 4 days earlier with SRBC.
† Duplicate spleen cell ($10^7$) cultures from two mice were set up on same day from all six groups. Mean ± SEM from two different experiments.

## RESULTS

**Influence of Dietary Restriction on Longevity.** In the present studies, our prior findings (20) of increased longevity in mice fed a diet of 10 cal/day compared to mice fed 20 cal/day were confirmed.

By age 400 days, 15 of the 15 female mice fed 20 cal per day had died, whereas only 4 of the 17 female mice fed 10 cal per day had died. With male mice, by 400 days of age, 7 of the 13 in the 20 cal per day group and 0 of the 11 in the 10 cal per day group had died.

**Influence of Dietary Restriction on Cell-Mediated Immunity.** Killer cell activity of 4-month-old mice fed the two diets was not different (Table 1). However, a much lower cell-mediated lysis was seen in 10-month-old mice fed the normal calorie diet than in mice of the same group fed the low-calorie diet. Greater variation between individual mice was observed in the older mice immunized with EL-4 cells *in vivo* while on the low-calorie diet.

Data are also presented in Table 1 which compare the *in vitro* generation of spleen killer cell activity against EL-4 lymphoma cells for B/W mice on either high or low calorie intake at 4 and 10 months of age. Again, the group on the low-calorie diet did not show the decline of *in vitro* T-cell-dependent immune response that occurred with aging in mice fed the high-calorie diet.

***In Vivo* and *In Vitro* PFC Responses.** After *in vivo* immunization, the number of PFC to SRBC generated at 3 months of age was lower in calorie-restricted mice when calculated per $10^6$ cells or per spleen (Table 2). A striking difference for PFC was seen in 10-month-old mice. A significantly depressed re-

Table 3. Influence of dietary intake on spontaneous suppressor cell activity in 3- and 10-month-old B/W mice

| Cells added $1 \times 10^6$ | Diet, cal/day | *In vitro* PFC | |
|---|---|---|---|
| | | Per $10^6$ cells | Per culture |
| Nil | *Ad libitum* | 146 | 1242 |
| | At 3 months old | | |
| Control | 20 | 250 | 1300 |
| Activated | 20 | 40 | 320 |
| Control | 10 | 200 | 860 |
| Activated | 10 | 80 | 492 |
| | At 10 months old | | |
| Control | 20 | 53* | 310* |
| Activated | 20 | 67 | 440 |
| Control | 10 | 442 | 2830 |
| Activated | 10 | 71 | 410 |

In each case, $10^7$ spleen cells from mice fed normal laboratory chow were the test culture. "Control" were cells precultured for 36 hr without Con A. "Activated" were cells precultured for 36 hr with Con A.

* A similar type of inhibition was observed in two other independent experiments.

sponse was observed at 10 months in mice given normal calorie diets, whereas those given low-calorie diets had better preservation of PFC at this age.

Table 2 also summarizes data comparing the number of PFC generated in spleen cell cultures after *in vitro* spleen cell stimulation with SRBC from mice fed either high- or low-calorie diets. In contrast to the lower numbers of PFC derived from *in vitro* stimulation, spleen cells from 4- and 6-month-old mice fed the low-calorie diets developed higher numbers of PFC by *in vitro* stimulation than those obtained from mice on the high-calorie diets. This was the case whether calculations were per $10^6$ cells or per culture dish, which initially contained $10^7$ cells. At 10 months of age, when a marked decline had occurred in the response of spleen cells of mice on the high-calorie diet, spleen cells from animals on the low-calorie diet still showed a high response to SRBC in the *in vitro* assay.

**Spontaneous Suppressor Cell Activity.** Table 3 presents data for the PFC response of spleen cells, cultured *in vitro* with SRBC, from 3-month-old mice fed normal laboratory chow. To these SRBC-stimulated cultures were added spleen cells from animals on either high- or low-calorie diets, which had been cultured previously for 36 hr either with medium alone or with Con A in the medium. (This system permits analysis of helper and/or suppressor influences generated spontaneously in media or after Con A stimulation *in vitro.*) When cells from 3-month-old mice fed the high-calorie diet were precultured in medium alone, there was a modest helper effect. A significant suppressor influence by Con A-stimulated spleen cells from mice of this age fed the high-calorie diet was observed. By 10 months of age, a change from helper to suppressor influence had occurred in both control cultures and Con A-stimulated cells of the high-calorie mice. These animals had generally already developed severe renal lesions (see below) by the time of this analysis. Spleen cells cultured from 3-month-old mice given the low-calorie diet responded similarly in this assay to those of 3-month-old mice fed a diet higher in calories. The mice fed only 10 cal per day, however, consistently showed helper rather than suppressor effects after preculture with medium alone. As was the case with cells from younger animals of both groups, spleen cells from the 10-month-old mice fed low-calorie diets developed suppressor activity after preculture with Con. A. Thus, calorie restriction appeared to inhibit development of spontaneous suppressor lymphocytes and to maintain a lymphocyte population that exerted a helper effect at 10 months of age. Lymphocytes from these animals could, however, be stimulated with Con A to exert a suppressor influence.

Table 4. Changes in binding of $^{125}$I-labeled DNA by sera from normal and calorie-restricted B/W mice*

| Diet, cal/day | % DNA binding at different ages | | |
|---|---|---|---|
| | 3 months | 6 months | 9 months |
| 20 | 8.4 ± 2.7 | 18.1 ± 4.7 | 53.2 ± 6.0 |
| 10 | 5.2 ± 0.7 | 11.6 ± 6.9 | 21.9 ± 2.8 |
| *P* | NS | NS | <0.001 |

* Each group consisted of five to eight mice. Data shown as mean ±SEM.

**Anti-DNA Antibodies.** Serum DNA-binding antibody levels were low in both groups at 3 months of age (Table 4). They increased slowly in mice fed the low-calorie diet and much more rapidly in those in the high-calorie group. By 9 months of age, the difference between the two groups was highly significant: the mice on the low-calorie intake had little anti-DNA antibody in their blood; those on high-calorie intake had significantly higher amounts of anti-DNA antibody.

**Histology and Immunofluorescence Microscopy.** Detailed studies of the light and immunofluorescence microscopic analysis of the renal lesions in the mice on the two different diets will be the subject of a separate report. For the sake of completeness we present, in this report, only representative observations concerning these parameters. Mice on the high-calorie intake showed advanced renal disease with extensive glomerular sclerosis, wire loop lesions, and cellular proliferation (Fig. 1*A*), whereas mice on the low-calorie intake showed little glomerular proliferation and virtually no wire loops or evidence of glomerulosclerosis (Fig. 1*B*). Animals on the high-calorie intake showed extensive capillary loop deposits and smaller mesangial deposits of IgG in the glomerular tufts (Fig. 1 *C*). The immunoglobulin deposits in the kidneys of mice on restricted calorie intake were, by contrast, confined largely to the mesangium (Fig. 1*D*). These observations indicate that the low-calorie diet protects against the renal lesions characteristic of B/W mice.

## DISCUSSION

In this study we confirm and extend our prior observations that showed that restriction of food from the time of weaning greatly prolongs life in the short-lived, autoimmunity-prone B/W mice (10). These animals, when fed a low-calorie diet (10 cal per day), grew more slowly than animals fed laboratory chow *ad libitum* or a normal calorie diet (20 cal per day). Nonetheless, the animals fed a low-calorie diet were active, appeared healthy, and showed no evidence of illness that might be attributed to severe malnutrition. However, parameters of lymphocytes responses and functions were affected differently by the calorie restriction. Phytohemagglutinin and Con A proliferative responses were generally greater in undernourished B/W mice up to 10 months of age, and responses to the B cell mitogen LPS were not significantly influenced by the low-calorie diet (unpublished data). The PFC response to *in vivo* immunization with SRBC was lower in calorie-deprived mice at a young age but remained higher at 10 months of age. Spleen cell responses to both *in vitro* and *in vivo* immunization against EL-4 cells to generate killer T lymphocytes and responses to *in vitro* immunization with SRBC to generate PFC were better preserved with aging in the

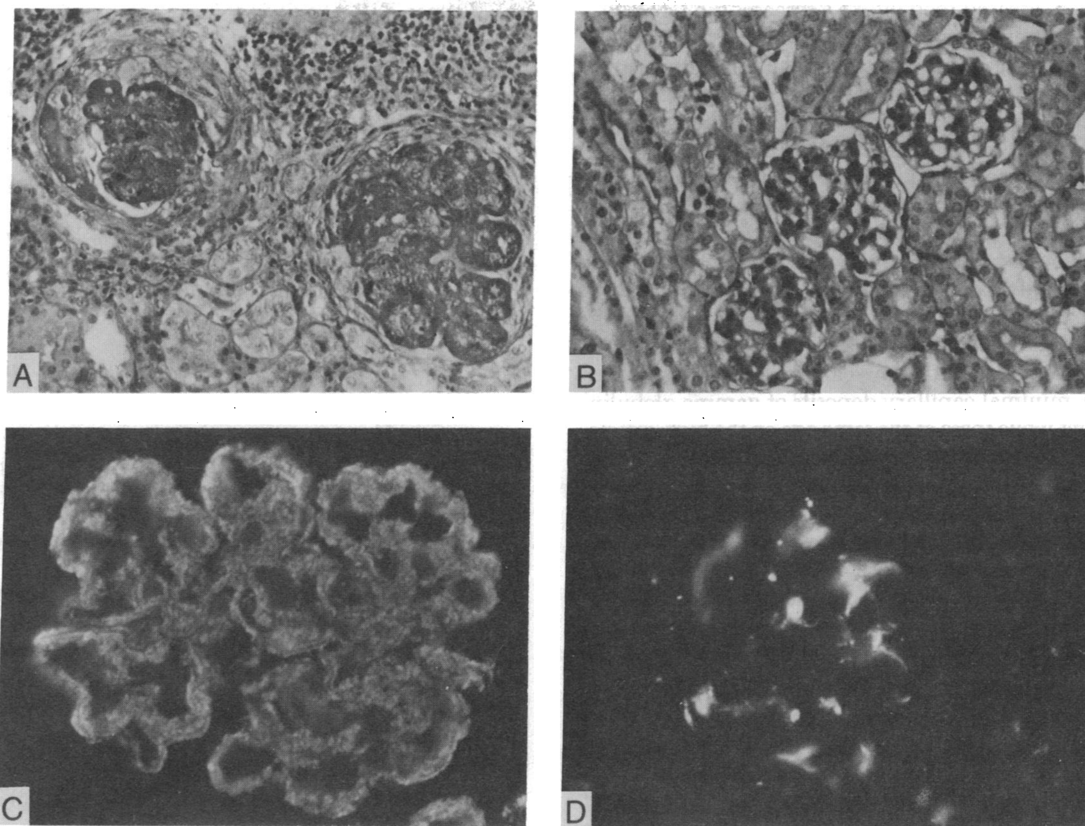


FIG. 1. (*A* and *B*) Histologic sections. (*A*) Typical glomerulus from a B/W mouse fed 20 cal per day. The glomerular tufts show proliferation and sclerosis. Note wire loop lesions similar to those seen in human lupus. (Hematoxylin and eosin; ×200.) (*B*) Typical glomerulus from mouse fed 10 cal per day. Note the well-preserved glomerular architecture and the absence of sclerosis, wire loop lesions, or evidence of proliferation. Minimal increase of mesangial cells is the only abnormality present. (Hematoxylin and eosin; ×250.) (*C* and *D*) Immunofluorescence microscopy. (*C*) Glomerulus of 10-month-old 20-cal per day B/W mouse stained with fluorescent antiserum specific for IgG. Note irregular granular deposits of IgG lining cell capillaries of the glomerulus. Every glomerulus of the kidneys of these older B/W hybrids showed these characteristics. (×500.) (*D*) Glomerulus of 10-month-old B/W mouse fed 10 cal per day. Note that glomerular capillaries are completely free of deposits of IgG. The only IgG demonstrable was present in the mesangium of the glomerular tuft. (× 650.)

mice fed low-calorie diets. Whether the lower PFC response to *in vivo* immunization with SRBC, observed with spleen cells of 3-month-old B/W mice on the calorie-restricted diet, is due to the influence of increased numbers of suppressor cells or to some other mechanism remains to be determined. For example, our previous results (24) in C3H mice, in which mammary adenocarcinoma was decreased by calorie restriction, showed increased activity of another kind of suppressor cell influence at 3 months of age.

One of the most impressive influences of the low-calorie diet in this animal model (B/W) system was the preservation, during aging, of the relationship between suppressor and helper cell influences that were present when the animals were young. In the mice fed the normal caloric intake, cells capable of exerting a strong suppressive influence on generation of PFC *in vitro* appeared in the spleens by 10 months of age. This development seemed to be dramatically inhibited by the lower calorie intake. In recent years, several studies have indicated that suppressor cells play a prominent role in regulation of the immune response to both soluble and cellular antigens (25). Whether these are the same cells that exert suppressor influences in old mice of other strains (26, 27) or in tumor-bearing animals (28) needs to be defined. The exact relationships of these influences on suppressor and helper functions in this animal model to the prolonged maintenance of T-cell immune functions and responses, anti-body production, and PFC responses *in vivo* and *in vitro* are not yet clear.

The present finding that B/W mice on normal diets develop a spontaneous, nonspecific suppressor cell influence agrees with original observations in older B/W mice reported by Roder *et al.* (29). On the other hand, previous findings have suggested that a loss of suppressor T-cell activity occurs at an earlier age in NZB and B/W mice (30–32) and that the autoimmune disease most likely develops in NZB mice because of an early loss of regulatory or suppressor T cells. In our present study, increased spontaneous suppressor cell activity was present in well-fed animals. These mice showed decreased PFC formation against SRBC but developed increased serum anti-DNA antibody. Absence of such suppressor cell activity in calorie-deprived animals, although facilitating PFC response, was associated with decrease of anti-DNA antibody as well as less evidence of antigen–antibody complex deposition in the kidneys. Therefore, it is possible that the increase in suppressor cells at a later age in well-fed animals is inhibiting a helper T-cell function essential to production of antibody against exogenous antigen but is not inhibiting B-cell function in forming autoantibodies. As to the influence of diet on lymphocyte function, it seems most pertinent to investigate this influence on the number and functional activity of lymphoid cells of different Ly phenotypes as defined by Shiku *et al.* (33), and Cantor *et al.* (34), especially in long-lived strains of mice.

Recent experiments have shown that the life-span of B/W mice can be prolonged by hypertransfusion or treatment with actinomycin D (35). It is possible that, as has been proposed for

actinomycin D, decreased food intake decreases the DNA delivery to catabolic pathways and could in this way have been responsible for slowing the progression to autoimmunity and progressive renal disease. From the present studies it is clear that calorie restriction lowers antibody titers to DNA below those seen in the B/W mice fed high-calorie diets. Major differences in degree of renal disease were also observed between mice fed the two diets. Those fed the low-calorie diet showed no evidence of glomerulosclerosis, hyalinization of glomeruli, or development of wire loop lesions at a time when these pathological changes were far advanced in the mice fed either laboratory chow *ad libitum* or the higher calorie diet. Correlated with these changes was the finding, by immunofluorescence microscopy, that the mice fed the low-calorie diet showed, at 10 months of age, minimal capillary deposits of gamma globulin and complement while those of the same age on the high-calorie intake showed extensive deposits of these immunologic components in the glomerular capillaries.

Needed, in addition to the titers of anti-DNA reported herein, are determinations of the amount of various forms of DNA found in the blood throughout life as well as analysis of the physical nature of antigen–antibody complexes present. Finally, it is important to elucidate the exact mechanism underlying the decrease of anti-DNA antibody and the reduced damage to the kidney which may play a most important role in prolonging the life-span of B/W mice. It seems imperative to understand whether the improvement of cell-mediated and humoral immunity exerts a major influence on delaying the appearance and progression of the autoimmune disease. From studies of the frequent association of autoimmunity with aging in other systems and with many primary immunodeficiencies of man, this certainly seems likely (36).

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